Iran. J. Radiat. Res., 2010; 7 (4): 211-216

Evaluation of combination effects of 2-methoxyestradiol and methoxyamine on IUdR-induced radiosensitization in glioma spheroids

A. Neshasteh-Riz[1*], S. Babaloui[2], S. Khoei[2]

[1]Department of Radiology, Iran University of Medical Sciences, Tehran, Iran
[2]Department of Medical Physics, Iran University of Medical Sciences, Tehran, Iran

Background: Glioblastoma is the most common and most malignant cancer of central nervous system. Targeted radiotherapy is an effective method toward its treatment. Iododeoxyuridine (IUdR) is a halogenated thymidine analogue known to be effective as a radiosensitizer in human cancer therapy. In this study we have evaluated the combination effects of 2-Methoxyestradiol, an inhibitor of hypoxia inducible factor 1α (HIF-1α) and Methoxyaminem, an inhibitor of base excision repair (BER) pathway on radiosensitization of IUdR in glioblastoma spheroid culture. ***Materials and Methods:*** The cytotoxic damages of DNA in U87MG cell line were compared using colony formation assay. Experiments were performed in large spheroids with a diameter of approximately 350µm. ***Results:*** Evaluation of the effects of IUdR with 2ME2 and MX pretreatment on spheroid cultured cell followed by ionizing irradiation showed more enhancemented ($p \leq 0.001$) IUdR induced-radiosensitization. These results introduced a key role for 2ME2 in IUdR related studies. ***Conclusion:*** Pretreatment of tumor cells with IUdR, MX and 2ME2 before irradiation enhances tumor radiosensitization and may improve therapeutic index for IUdR and 2ME2. **Iran. J. Radiat. Res., 2010; 7 (4): 211-216**

Keywords: *Hypoxia-inducible factor-1α, IUdR, radiosensitization, spheroid, methoxyamine, 2-methoxyestradiol.*

INTRODUCTION

Malignant gliomas are the most common adult primary brain tumors, occurring at a rate of five out of 100000 human populations a year (1). Normally, surgery fallowed by radiotherapy is the first treatment strategy (2); however, normal tissue tolerance is the most important obstacle against solid tumor radiation therapy as well as gliomas (3).

In recent years, combine treatments using chemical/biological agents and radiotherapy have been employed to either increase tumor radiosesitivity, or diminish ionizing radiation side effects. Iododeoxyuridine (IUdR) is a thymidine analoge, known as a potencial radiosensitizer for human cancers therapy. It incorporates into DNA instead of thymidine during replication and sensitizes the cells to ionizing radiation. Although the biochemical mechanism of IUdR induced radiosesitivity is not understood, it is presumed that IUdR sensitizes the cells through enhancing formation of DNA single and double strand breaks (4, 5) where the extent of radiosensitization correlates with the level of IUdR-DNA incorporation (6, 7).

Like solid tumors, the multicellular spheroid cultures represents cell-cell contact (8, 9), individual hypoxic cell populations (10), and cycle times that range from as those of exponential monolayer rates through an essentially non dividing state (11). A research conducted on the growth of human glioma cells in these two systems showed different degrees of sensitivity to radioionated IUdR (12). Several authors have reported higher radioresistance of cells in spheroids compared with monolayer cultures (13, 14). We have also found out that the DNA damages are strongly diminished in large volume spheroids in comparison with the small one which has been due to the existence of G_0/hypoxic cells in large spheroids unabling to absorb IUdR (15). So the application of these data in tumors including hypoxic/G_0 arrested cells, with no

****Corresponding author:***
Dr. Ali Neshasteh Riz,
Department of Radiology, Iran University of Medical Sciences, P.O. Box: 14155-6183, Tehran, Iran.
Fax: *+ 98 21 88054355*
Email: *neshastehriz@yahoo.com*

DNA replication, leads to deficiency in IUdR uptake and therefore inability to induce radiosensitivity consequently. Recent data have shown that 2-Metoxyestradiol (2ME2) inhibits hypoxia inducible factor-1α (HIF-1α). HIF-1α is a transcriptional activator that functions as a master regulator of cellular and systemic oxygen homeostasis [16]. 2ME2 is an endogenous metabolite of estrogen that has both angiogenic and antitumor effects. The ability of 2ME2 to inhibit HIF-1α correlates with its microtubule depolymerization effects [17]. So the present study was designed to search on radiosensitization effect of IUdR as a radiosensitizer and MX plus 2ME2 as BER and HIF-1α inhibitors, respectively. The experiment was done on U87MG cells, cultured in spheroid culture model. This study was performed on 350μm spheroids regarding the fact that G_0 cells were mostly in the same spheroids size.

One of the well known and reliable methods in radiobiological studies is colony formation assay. Colony formation assay is an *in-vitro* cell survival assay based on ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo unlimited division. Colonogenic assay which is used in this study is the method to determine cell reproductive death after treatment with ionizing radiation. It can also be used to determine the effectiveness of other cytotoxic agents [18].

MATERIALS AND METHODS

Cell line

Human glioblastoma cell line U87MG was provided by Pastor Institute of Iran. This cell line was maintained in MEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 500U/ml of penicillin/ 200 mg/lit of streptomycin (SIGMA).

Monolayer culture

Cells were cultured as monolayer at a density of 31250 cells/ml in T-25 tissue culture flasks (NUNC). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cultures were propagated and cells were harvested by trypsinizing cultures with 1mM EDTA/ 0.25% Trypsin (w/v) (SIGMA) in Phosphate Buffer Saline (PBS).

Spheroid culture

Spheroids were initiated using the Liquid Overlay technique [19]. Cells were seeded into100 mm plates coated with a thin layer of 1% agar (Bacto Agar, Difco, Detroit, MI) with 10ml of MEM supplemented with 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Half of the culture medium was replaced with fresh medium twice per week.

Drug-radiation treatment

The 350μm diameter formed spheroids were treated with IUdR, MX, 2ME2 67h before being exposed to ionizing radiation. The concentration of the chemical was 1μM, 6mM and 250μM, respectively, in MEM containing 10% FBS. After the treatment time, the medium containing drugs was removed and the cultures were washed 3 times with PBS and the spheroids were immediately irradiated using ^{60}Co source (Theratron 760) at a dose rate of 109.29c Gy/min for 2 Gy. For radiation treatment, 4 tissue culture flasks were put under collimator of equipment at 65cm distance of the head simultaneously, and the field size and the period of irradiation were 20×10 cm^2 and 1.83min, respectively.

Colony formation assay

After drug treatment and irradiation, to achieve single cells, spheroids were trypsinized with 300 μl of trypsin in 5 minutes. Then, single cells plated for colony formation tests. In day 10 after plating, cultures washed 3 times with PBS, fixed with 2% formaldehyde in PBS for 15 min, stained with 0.5% crystal violet, and colonies were counted by invert microscope

(Olympus).

In order to evaluate the ability of cells to form colonies, different concentration of individual cells (2000, 3000, 5000, 7500, 10000 cells) from spheroid seeded into 60mm plates with 10ml of MEM supplemented with 10% FBS. Plating efficiency were calculated, using the following equation:

PE (%) = (the number of colonies / the number of seeded cells) ×100

Statistical analysis

Data were given as mean values±SEM, with "n" denoting the number of experiments. One way Anova (Analysis of Variance, non parametric) was considered appropriate and applied. A value of $p \leq 0.05$ was considered to be significant.

RESULTS

The U87MG cells were able to form spheroids in liquid overlay cultures. Figure 1 shows the phase contrast micrograph of the spheroid in 350 µm in diameter. The volume doubling time (VDT) calculated from the U87MG spheroid was ~67±0.91h [15] which was applied for drug treatment time, consequently.

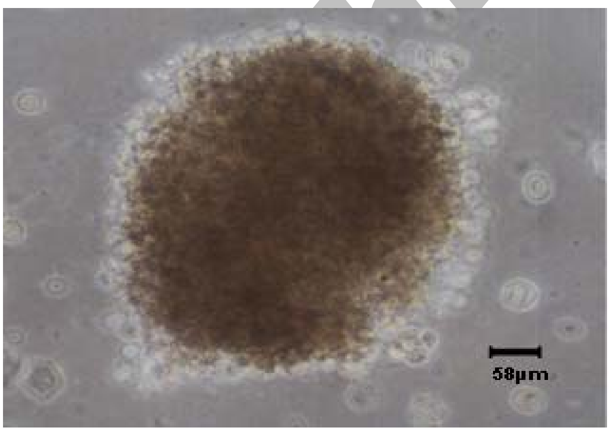


Figure 1. Phase contrast micrographs of U87MG spheroids. 350µm spheroid on the day 24th after initiation of measuring the diameters.

In the first treatment time, i.e. 67±0.91h, the spheroids were treated with 2ME2 to inhibit HIF-1α activities producing until the formation of 350µm spheroids. Then, spheroids were treated with IUdR+MX, IUdR+2ME2 and IUdR+MX+2ME2 for next VDT. Finally, after drug treatment and irradiation, the numbers of colonies were counted for the evaluation of radiosensitization effects. The average of maximum and minimum of numbers of formed colonies were 150 and 17, respectively.

Figure 2 shows a cell population containing more than 50 cells which indicating a standard colony of U87MG cell. Based on the results of colony formation of different pre-cultured cells, the optimum cell number to perform colony from spheroids was ~5000 per 60 mm dishes. The results are shown clearly in figure 3.

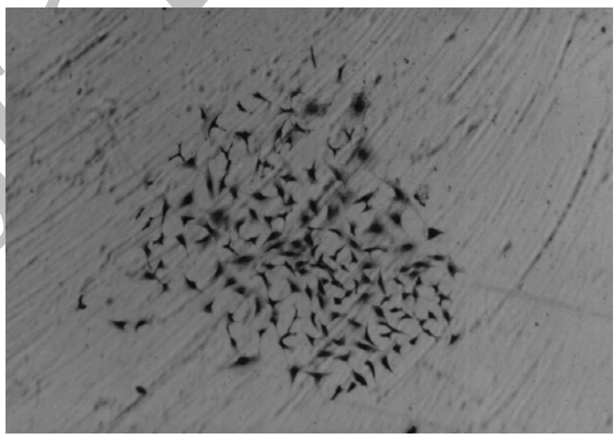


Figure 2. A colony formatted by an invert microscope of U87MG cell line.

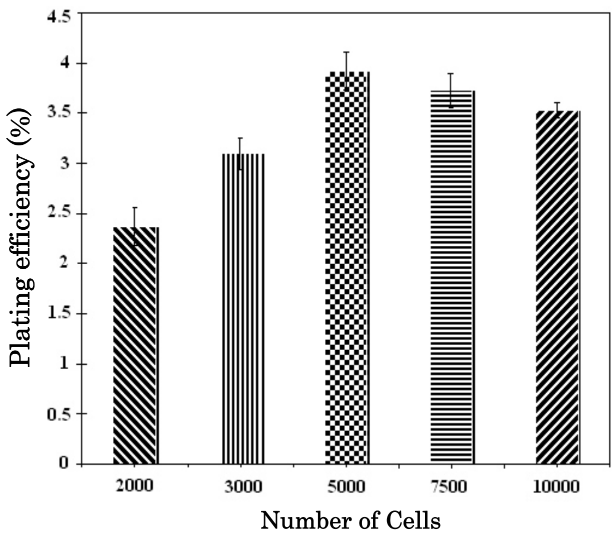
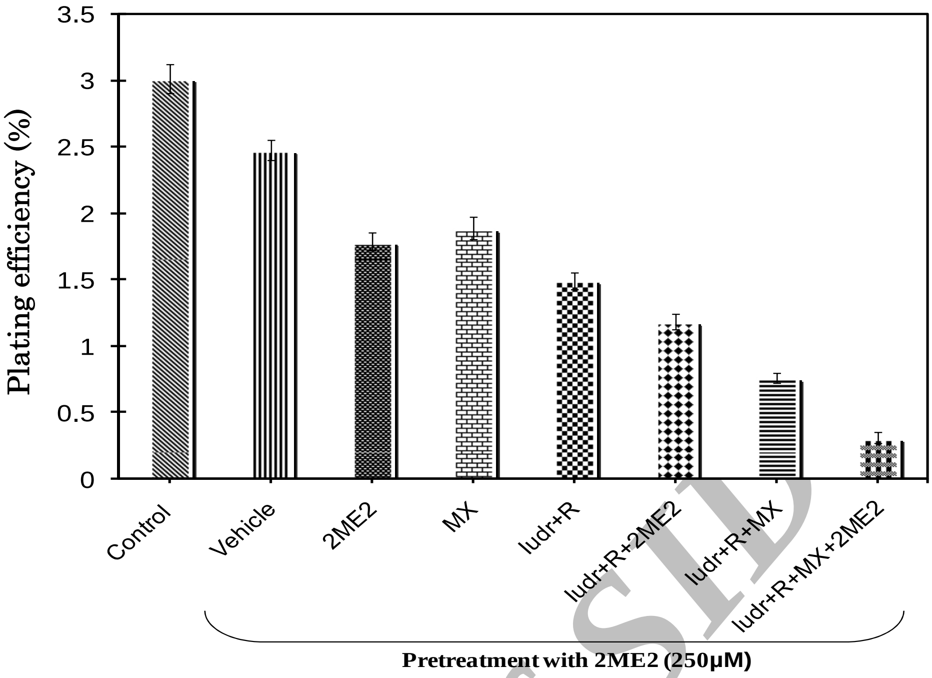


Figure 3. Plating efficiency using U87MG cells of spheroid culture in MEM supplemented with 10% FBS in different concentrations. Mean ± SEM of 3 experiments.



According to figure 4, colonies formed from the control and experimental groups were significantly decreased ($p<0.001$). In the third group, 2ME2 treated cultures, were damaged significantly ($p<0.001$), and the increase was more or less the same as MX results treated. Consequently, IUdR/MX and IUdR/2ME2 treatments showed more damages (less colonies) ($p<0.005$) in comparison with the control group which was treated without IUdR. There was also a big difference, in IUdR/MX/2ME2 group in comparison with the control as well as other groups ($p<0.001$).

DISCUSSION

Methoxyamine (MX) as a BER inhibitor, shown to mediate by tight binding to AP sites generated by cleavage of BER glycosylases and rendering the phosphodiester bonds adjacent to the AP site refractory to the catalytic activity of AP endonuclease [20, 21], resulting well blockage of BER pathway. In our previous study, it has shown that MX increased DNA damages thorough enhancing of DNA strand breaks in spheroid cultures of U87MG cell lines [15]. However IUdR with MX radiosensitization studies, are restricted to *in-vitro* researches on monolayer culture cells from human colon cancer [20-22]. Therefore, first; it remains unclear whether these results could be extended into tumor *in-vivo*; second, based on high proliferation property of brain tumors, which makes them a good candidate for IUdR applications, is it possible to achieve the same results obtained from colon cancer cells. To answer these two important questions, we have studied the radiosensitization effects of IUdR/MX on glioma spheroid cell culture.

Spheroid cultures are interesting and valuable *in-vitro* model systems which allow many properties of *in-vivo* tumor systems to be studied quantitatively [9-11]. Tumor hypoxia may be back to hypoxia inducible factor-1α (HIF-1α) which is found in mammalian cell cultured under reduced O_2 tension (23). Recent studies have shown that HIF-1α is responsible for arresting endothelial cells at G_0 phase [16], resulting in the reduced proliferation of cells and the hypoxia-induced death may be involved by suppression of anti-apoptotic molecule, bcl-2 [14,16,23]. Studies have also demonstrated that increasing the size of DU-145 prostate multicellular tumor spheroids decreases the pericellular oxygen pressure and generation of reactive oxygen species, whereas the α-subunit of HIF-1 is up-regulated (24).

Furthermore, over-expression of HIF correlates with metastasis decreased response to radiation and chemotherapy. Recent data have shown that 2ME2 inhibits HIF-1α in cancer cell lines. 2ME2-mediated decrease in cellular HIF-1α levels leads to inhibition of its nuclear translocation, inhibition of VEGF transcription and a decrease in VEGF secretion. Over 60 genes have been identified as HIF-1 target genes. Under hypoxic condition 2ME2 down-regulates HIF-1α protein levels in human umbilical vein endothelial cells, as well (17, 25). In this study, we hypothesized that inhibition of HIF-1α using 2ME2 would be effective in IUdR/MX radiosensititation within blockage of G_o arrests and consequently, in IUdR uptake in glioma spheroids.

Using colonogenic assay (figure 4), vehicle has shown significant more cell damages in comparison with control, which might have been due to pretreatment with 2ME2 resulting to cell apoptosis. Also, the result showed that IUdR pretreatment sensitized cells to ionizing radiation significantly, and the degree of radiosensitization was further increased, when cells were pretreated with IUdR/MX and IUdR/2ME2 in comparison with control. In IUdR/MX pretreatment group first: MX increased IUdR-DNA incorporation which resulted in IUdR-induced radiosensitization, and second: MX inhibited short-patch BER by bloking APE activity that increase SSBs and DSBs generated during BER. MX stabilized AP sites and blocked cleavage of the phosphodiester bond which reduced the BER intermediate. In IUdR/2ME2 pretreatment group, first: 2ME2 inhibited expression, and activation of HIF-1α proteins, cells passed synthesis phase, uptook IUdR and sensitized to ionizing radiation, and second: 2ME2 inhibited cell cycle progression in the G_2-M phase via disruption of microtubule elongation. As we know, G_2-M phase is a radiosensitive phase of the cell cycle, thus 2ME2 also acts as a radiosensitization. Besides, IUdR/MX/2ME2 have the most radiosensitization effect comparing with control and the other groups. However, these favorable results need a comprehensive molecular biology experiments. According to our results and former studies outcome, the HIF-1α, as a responsible protein for G_o/G_1 arrest, inhibited by 2ME2 and, therefore, increase cell proliferation and IUdR-DNA incorporation. HIF-1α as a key factor to open a gate for IUdR related studies at least in brain tumors, may improve therapeutic index for clinical radiation.

REFERENCES

1. Laperriere N, Zuraw L, Cairncross G (2002) The cancer care ontario practice guidelines institute neuro-oncology disease site group. Radiotherapy for neely diagnosed malignant glioma in adults: a systematic review. *Radiotherapy and Oncology, **64:** 259-273.*
2. Jemal A, Murray T, Samuels A, Chafoor A, Ward E, Thun MJ (2003) Cancer statistics. *CA Cancer JCLIN, **53:** 5–26.*
3. Scheline GE, Wara W, Smith V (1980) Therapeutic irradiation and brain injury. *Int J Radiant Oncol Biol Phys, **6:** 1215-1228.*
4. Fornace AJ Jr, Dobson PP, Kinsella TJ (1990) Enhancement of radiation damage in cellular DNA following unifilar substitution with iododeoxyuridine. *Int J Radiat Oncol Biol Phys, **18:** 873-8.*
5. Watanabe R and Nikjoo H (2002) Modelling the effect of incorporated halogenated pyrimidine on radiationinduced DNA strand breaks. *Int J Radiat Biol, **78:** 953-966.*
6. Miller EM, Fowler JF, kincella TJ (1992) Lirear-quadratic analysis fo radio sensitization by halogenated pyrimidines. I. Radio sensitization of human colon cancer cells by iododeoxy uridine. *J Radiat, **9131:**81-89.*
7. Lawrence TS, Davis MA, Maybaum J, Stetson PL, Ensminger WD (1990) The dependence of halogenated pyrimidine incorporation and radiosensitization on the duration of drug exposure. *Int J Radiat Oncol Biol Phys, **18:** 1393-1398.*
8. Gene M, Casrro Kreder N, Barten-van Rijbroek A, Stalpers KJ, Haveman J (2004) Enhancement of effects of irradiation by gemcitabine in a glioblastoma cell line and cell line speroids. *J Cancer Res Clin Oncol, **130:** 45-51.*
9. Santini MT, Rainaldi G, Indovina PL (2000) Apoptosis, cell adhesion and the extracellular matrix in the three dimensional growth of multicellular tumor spheroids. *Crit Rev Oncol Hematol, **36:** 75-87.*
10. Woods ML, Koch CJ, Lord EM (1996) Detection of individual hypoxic cells in multicellular spheroids by flow

cytometry using the 2-nitroimidazole, EF5 and monoclonal antibodies. *Int J Radiat Oncol Biol Phys, **34:** 93 -101.*

11. Durand RE and Olive PL (1992) Tumour cell kinetics and heterogeneity: insights from multicell spheroids. *BJR Suppl, **24:** 79-83.*
12. Neshasteh-Riz A, Mairs RJ, Angerson WJ, Stanton PD, Reeves JR, Rampling R, Owens J, Wheldon TE (1998) Differential cytotoxicity of [^{123}I] IUdR, [^{125}I] IUdR and [^{131}I] IUdR to human glioma cells in monolayer or spheroid culture: effect of proliferative heterogeneity and radiation cross-fire. *Br J Cancer, **77:** 385-390.*
13. Olive PL and Durand RE (1994) Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev, **13:** 121-138.*
14. Desoize B and Jardillier J (2003) Multicellular resistance: a paradigm for clinical resistance. *Crit Rev Oncol Hematol, **6:** 193-207.*
15. Neshaste-Riz A, Saki M, Khoei S (2008) Cytotogenic damages from iododeoxyuridine- induced radiosensitivity with and without metoxyamine in human glioblastoma spheroids. *J Yakhteh, **10:** 57-64.*
16. Iida T, Mine SH, Fujimoto H, Suzuki K, Minami Y, Tanaka Y (2002) Hypoxia- inducible factor-1α induces cell cycle arrest of endothelial cells. *Genes to Cells, **7:** 143-149.*
17. Mooberry SL (2003) Mechanism of action of 2-methoxyestradiol: new developments. *Drug Resistance Updates, **6:** 355-361.*
18. Franken N AP, Rodermond HM, Stao J, Haveman J, Bree CH V (2006) Clonogenic assay of cells *in-vitro*. *Nature Protocols, **1:**2315-2319.*
19. Wigle JC and Sutherland RM (1985) Increased thermoresistance developed during growth of small multicellular spheroids. *J Cell Physiol, **122:** 281-289.*
20. Taverna P, Hwang HS, Schupp JE, Radiovoyevitch T, Nguyen Session N, Reddy G, Zarling DA, Kinsella TJ (2003) Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. Cancer Res, 63: 838-846.
21. Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, Kinsella TJ (2006) Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. *Mol Cancer Ther, **5:** 893-902.*
22. Liu L, Nakatsuru Y, Gerson S L (2002) Base excision repair as a therapeutic target in colon cancer. *Clin Cancer Res, **8:** 2985–2991.*
23. Wang GL, Jiang BH, Rue EA and Semenza GL (1995) Hypoxia- inducible factor1 is a basic halix-loop-halix heterodimer regulated by cellular O_2 tension. *J Proc Natl. Acad. Sci. USA, **92:** 5510-5514.*
24. Wartenberg M, Ling FC, Muschen M, Klein F, Acker H, Gassmann M, Petrat K, Putz V, Hescheler J, Sauer H (2003) Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor-1 and reactive oxygen species. *The FASEB Journal, **17:** 503-5.*
25. Ricker JL, Chen ZH, Yang XP, Pribluda VS, Swartz GM, Waes CV (2004) 2-Methoxyestradiol inhibits hypoxia-inducuble factor 1α, Tumor growth and angiogenesis and augments paxitaxel efficacy in head and nea; squamous cell carcinoma. *J Clinical Cancer Research, **10:** 8665-8673.*